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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Since collagenases (-1 and -3) degrade the extracellular bone matrix (ECM) components, collagenase-driven ECM proteolysis may facilitate cancer growth and progression. To test this hypothesis, we proposed to utilize a transgenic mouse model to overexpress collagenase-1 under the control of the bone specific osteocalcin promoter. The expression plasmid pCMV-collagenase-1-V5 was constructed, which contains an eukaryotic promoter (CMV) fragment and a 1.65 kb human collagenase-1 cDNA fragment, followed by a V5 epitope tag. The pCMV-collagenase-1-V5 was transiently transfected into COS-7 cells and the expression of collagenase-1-V5 was identified by Western blot using the anti-V5 antibody. The pOC-collagenase-1-V5 was constructed by replacing the CMV promoter sequence from pCMV-collagenase-1-V5 with the rat osteocalcin promoter sequence (OC). The ability of osteocalcin promoter to drive collagenase-1-V5 expression is being carried out by transient transfection assays and Western blot analysis. The 2.6 kb DNA fragment containing the osteocalcin promoter (1 kb) and collagenase-1-V5 cDNA sequence will be excised from pOC-collagenase-1-V5 with appropriate enzymes and used for generation of transgenic mice.
In addition, the molecular mechanism(s) responsible for transforming growth factor-β1 (a crucial molecule in metastatic bone cancer) stimulation of collagenase-3 expression in human breast cancer cells was investigated.

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Table of Contents

Collagenases in Breast Cancer Cell-induced Metastatic Tumor Growth and Progression:

Introduction:

Matrix metalloproteinases (MMPs) appear to play an important role in the multiple steps of breast cancer development and metastasis (1-4). Since collagenases-1 and -3 (MMP-1 and -13) are characterized by their potent ability to degrade the extracellular bone matrix (ECM), it is likely that collagenase-driven ECM proteolysis supports cancer cell expansion both biochemically by exposing mitogenic factors and physically by providing space for the proliferating cells. It is also possible that growth factors and cytokines released from the ECM could act in a feed-forward manner by inducing cancer cells to secrete more collagenases.

Bone is one of the major sites for formation of breast cancer metastases. The molecular mechanisms responsible for osteolytic metastases are complex and involve bi-directional interactions between tumor cells and bone. TGF-β1 (transforming growth factor--β1) is a crucial molecule in metastatic breast cancer. It is released as a result of bone resorption and may alter the nature of tumor cells, resulting in more aggressive local bone resorption and osteolysis. TGF-β1 can potentially disrupt the normal balance between osteoclast- and osteoblast-derived MMP activity within bone by inducing the expression of MMPs and their inhibitors from bone-metastasizing cancer cells.

Body:

In order to study the role of collagenases on cancer growth and progression, we proposed to utilize a transgenic mouse model to overexpress collagenase under the control of the bone specific osteocalcin promoter. The osteocalcin promoter has been shown to confer differentiated osteoblast- and post-specific expression to a reporter gene *in vivo*. To generate transgenic mice overexpressing collagenase, we first initiated our work to clone the human collagenase-1 cDNA (1.65 kb) and express it *in vitro*. We used pcDNA3.1 Directional TOPO Expression construct (Invitrogen) for this purpose. The pcDNA3.1 contains the following elements: human cytomegalovirus (CMV) immediate-early promoter/enhancer that permits efficient, high-level expression of recombinant protein and V5 epitope that allows detection of recombinant protein with anti-V5 antibody. The collagenase-1 cDNA followed by a V5-epitope tag was cloned downstream into the CMV promoter sequence. The construct pCMV-MMP-1-V5 was transfected into COS-7 cells using the Lipofectamine 2000 reagent (Invitrogen) according to the guidelines provided by the company. Cells were lysed and subjected to Western blot analysis. The results indicate that collagenase-1-V5 is efficiently expressed under the control of CMV promoter.

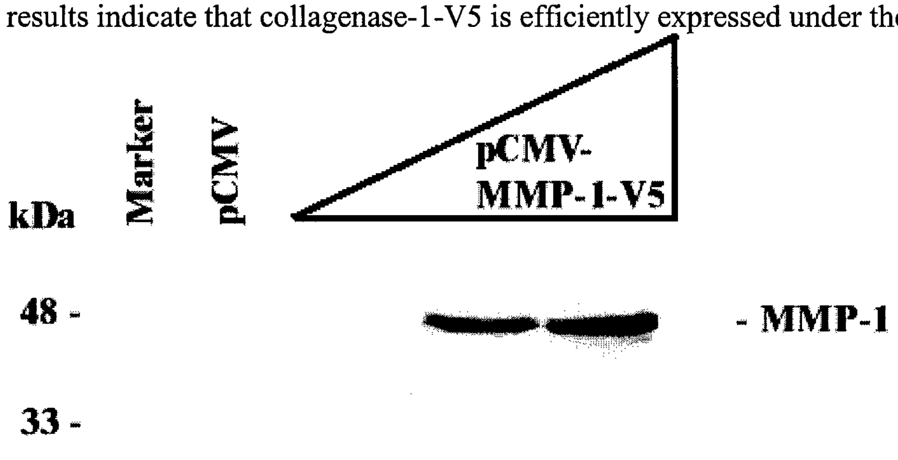


Figure 1. Expression of MMP-1 (Collagenase-1). The construct (pCMV-MMP-1-V5) at different concentrations was transfected into COS-7 cells using the Lipofectamine 2000 reagent for 24 h. The cells were then lysed. The lysates were subjected to 12% SDS polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride (PVDF) membranes and the immunoblot was probed with anti-V5 monoclonal antibody.

In order to get bone specific expression of collagenase-1, we utilized the rat osteocalcin promoter (OC; ~1.0 kb). It was kindly provided by Dr. Gary Stein, University of Massachusetts Medical School, Worcester, MA. The pOC-collagenase-1-V5 was constructed by replacing the CMV promoter sequence from pCMV-MMP-1-V5 with the rat osteocalcin promoter sequence. The ability of osteocalcin promoter to drive collagenase-1-V5 expression is being carried out by transient transfection assays and Western blot analysis. The 2.6 kbp DNA fragment containing the osteocalcin promoter and collagenase-1-V5 cDNA sequence will be excised from pOC-collagenase-1-V5 with appropriate enzymes and used for generation of transgenic mice.

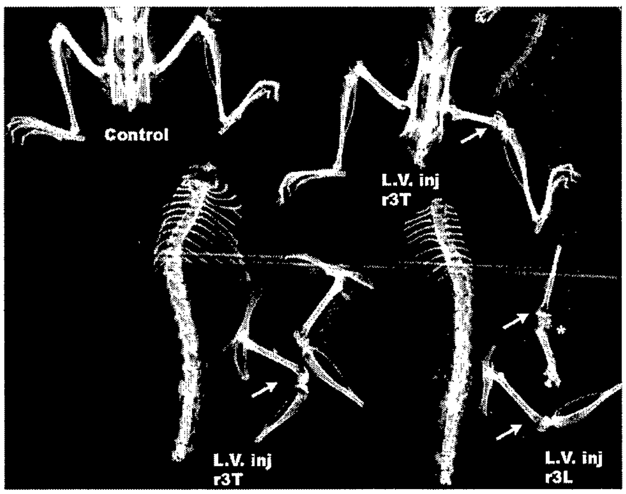
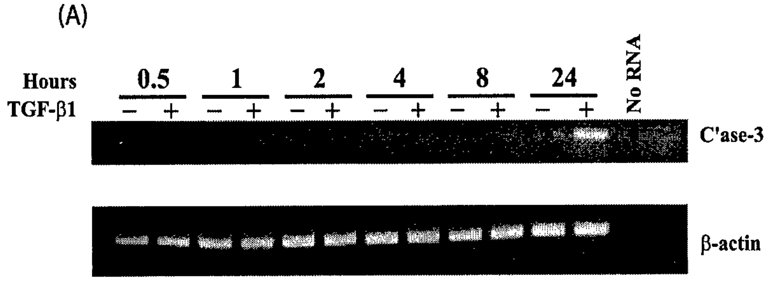


Figure 2. Cardiac injection and tumor analysis. My collaborator (Dr. Susan Rittling, Rutgers University, NJ) generated a series of metastatic murine mammary epithelial cell lines using normal mice rather than using nude mice. Mammary tumors were induced in strain 129 female mice by treatment with the carcinogen DMBA and their growth was accelerated by implanted progesterone (MPA) pellets. An epithelial cell line was isolated from one of these tumors: however, this cell line (1029) was not tumorigenic in mice even in the presence of MPA. Introduction of the oncogene polyoma middle T into these cells (1029 GP+E) was insufficient to allow hormone-independent tumor growth, although in the presence of hormone (MPA) rare tumors were observed. Thus, a second oncogene, v-Ha-ras was introduced. Cells expressing both ras and polyoma middle T were able to form tumors efficiently in the mammary fat pad, and even formed spontaneous metastases in the lungs and sometimes liver after mammary tumor growth (1029 GP+E r3). Two additional cell lines were derived from the 1029 GP+E r3 cells – one from a tumor and one from a lung metastasis arising from injected cells: these cells were called r3T and r3L, respectively. These two cell lines have similar metastatic properties. <u>To get expertise in the techniques of cardiac injection of cancer cells into the mice and tumor analysis,</u> we utilized those cancer cells with normal mice. The r3T or r3L cells (5×10^5) were injected into the left ventricle (L.V.) of the heart, and the mice were sacrificed three weeks later. Arrows indicate positions of extensive bone loss visible in these x-ray images. The bone indicated by an asterisk was actually broken completely as a result of tumor growth.

Collagenase-3 (MMP-13) is overexpressed in a variety of malignant tumors. In the present study we show that TGF-β1 stimulates collagenase-3 expression in the human breast cancer cell line MDA-MB231. Since collagenase-3 is characterized by its ability to degrade the ECM and is stimulated by TGF-β1 in MDA-MB231 cells, collagenase-3 driven ECM proteolysis may support cancer cell growth both biochemically by exposing mitogenic factors and physically by providing space for the proliferating cells. To delineate the molecular mechanisms responsible for this stimulation of collagenase-3 by TGF-β in these cells, a functional analysis of the collagenase-3 promoter was carried out.

TGF-β1 stimulation of collagenase-3 expression in MDA-MB231 cells requires *de novo* protein synthesis - To study the effect of TGF-β1 on expression of collagenase-3 in human breast cancer, MDA-MB231 cells were treated with TGF-β1 either at different concentrations for 24 h or for different time periods with TGF-β1 (10 ng/ml). Total cellular RNAs were purified and analyzed by semi-quantitative RT-PCR. TGF-β1 maximally stimulated collagenase-3 RNA expression at 10 ng/ml concentration in MDA-MB231 cells (data not shown) and requires at 24 h (Fig. 3A). The identity of collagenase-3 (392 bp) and β-actin (661 bp) PCR products were confirmed by transferring PCR products to filters and hybridizing to labeled human collagenase-3 and β-actin cDNA probes, respectively.

(A)



(B)

Treatment	Collagenase-3 secretion (ng/ml)
Control	1.29 ± 0.069
TGF-β1	4.22 ± 0.103[a]

(C)

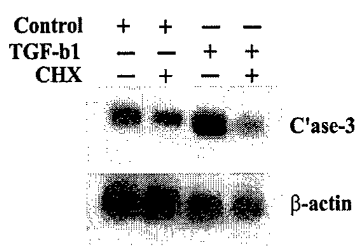


Figure 3. TGF-β stimulation of collagenase-3 expression in MDA-MB231 cells requires *de novo* protein synthesis. A, MDA-MB231 cells were serum starved for 24 h and then treated with TGF-β1 (10 ng/ml) at different time periods as indicated. Sense and antisense oligos for human collagenase-3 (C'ase-3) and β-actin were used for RT-PCR. **B,** MDA-MB231 cells were serum starved for 24 h and then treated with control medium or medium containing TGF-β1 (10 ng/ml) for 24 h. The collagenase-3 levels in the media were measured using an ELISA kit (Amersham Pharmacia). Data represent mean ± S. E. of three replicate plates. The statistical analysis was performed using Student's *t*-test and Prism 3.0. [a]Significant difference compared with control ($P< 0.001$). **C,** MDA-MB231 cells were serum starved for 24 h and then treated with control medium or medium containing TGF-β1 (10 ng/ml) for 24 h in the presence or absence of cycloheximide (30 μg/ml, added 1 h before TGF-β1 treatment). Total RNA was subjected to Northern blot analysis and probed with labeled human collagenase-3 or β-actin cDNA.

An ELISA was then performed using an antibody to human collagenase-3 confirming increased secretion of collagenase-3 into the medium from TGF-β1-treated MDA-MB231 cells (Fig. 3B). To determine if the TGF-β-mediated increase in collagenase-3 mRNA is a primary response, we used the protein synthesis inhibitor cycloheximide. As shown in Fig. 3C, cycloheximide inhibited TGF-β1 stimulation of collagenase-3 mRNA, indicating that *de novo* protein synthesis is required for this response.

The RD and AP-1 sites are necessary for TGF-β1-stimulated collagenase-3 promoter activity - The collagenase-3 promoter region includes consensus binding sites for several DNA-binding proteins, C/EBP, SBE (smad binding element), RD (runt domain binding sequence), p53, PEA-3, AP-2, and AP-1 (activator protein-1) (5). There are four consensus sites, namely a RD site, a p53 site, a PEA site, and an AP-1 site, which are highly conserved both in sequence and location in both the human and rat collagenase-3 promoters. The RD binding site is identical to a Cbfa/Runx binding site and the proteins binding to this site are Cbfa/Runx transcription factors (6-8). Previously, we deleted regions of the rat collagenase-3 promoter from the 5'-end and placed the resulting promoter sequences 5' of the CAT (chloramphenicol acetyltransferase) gene. We also showed that the AP-1 site is mainly involved in basal expression; whereas the RD site is necessary for PTH-induced collagenase-3 promoter activity in the rat osteoblastic cell line, UMR 106-01 (5). Both the RD and AP-1 sites are contained within the 148 base pairs upstream of the transcriptional start site. The collagenase-3 promoter also contains another RD site (proximal) overlapping with the AP-1 site and its sequences (ACCAC) are similar to the Cbfa/Runx consensus site, ACC(A/G)CA (9). Even though the AP-1 site is conserved among the human and rat collagenase-3 genes, the proximal RD site overlapping with the AP-1 site is located in the opposite orientation and on the opposite strand (Fig. 4A). Since it has been reported that the RD site acts as enhancer (10), the proximal RD site could also be a functional element in mediating the TGF-β-response.

In order to analyze the specific response elements involved in TGF-β1-stimulated collagenase-3 promoter activity in MDA-MB231 cells, the promoter constructs having mutations at either the distal RD or the proximal RD or the AP-1 sites were used and the effect on CAT activity was assessed in these cells. Mutation of either the distal RD or the proximal RD or the AP-1 sites reduced the basal activity; whereas mutation at any one of those sites was enough to cause a significant loss of TGF-β1-response for collagenase-3 promoter activity in the breast cancer cells (Fig. 4B). Hence, these results suggest that the distal RD and the proximal RD/AP-1 sites are necessary for full TGF-β1-stimulated collagenase-3 promoter activity.

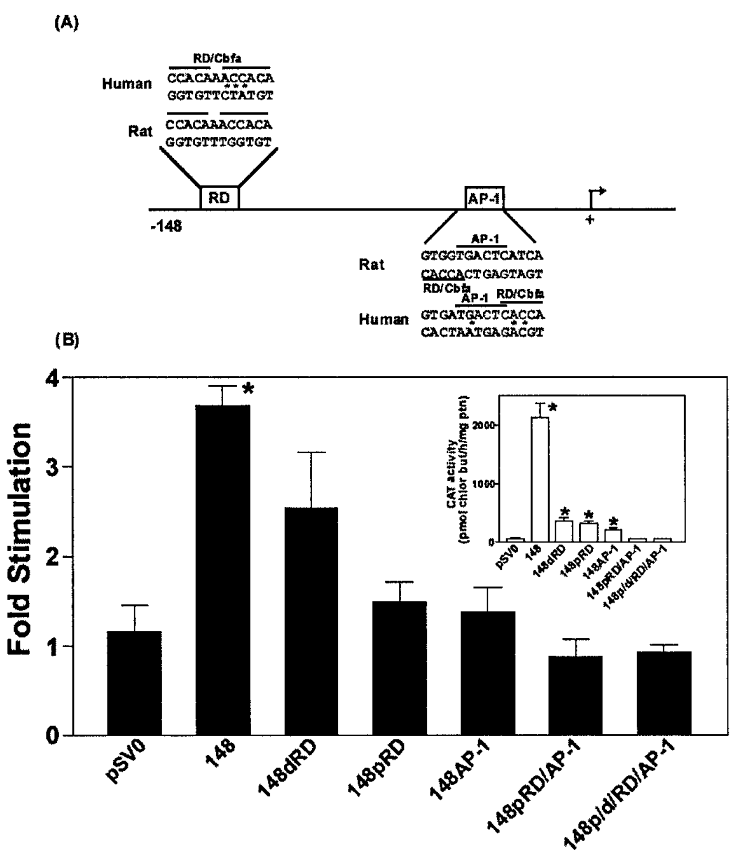


Figure 4. The RD and AP-1 sites are necessary for TGF-β1-stimulated collagenase-3 promoter activity. A, Comparison of the nucleotide sequence of the distal RD and the proximal RD/AP-1 sites of the –148 rat and human collagenase-3 promoters (+ indicates the transcription initiation site). The *asterisks* indicate mutated nucleotides within human collagenase-3 distal RD, AP-1, and proximal RD sites. **B,** The wild type and mutant rat collagenase-3 promoter constructs were transiently transfected into MDA-MB231 cells, treated with control or TGF-β1-containing media for 24 h, and then assayed for CAT activity. The TGF-β1-response is shown as -fold stimulation over control. The *inset* graph shows the basal activity of collagenase-3 promoter constructs. Data represent mean ± S.E. of three experiments. *Significant difference compared with control (promoterless) vector, pSV0 ($P < 0.05$). (dRD-distal RD site; pRD-proximal RD site; AP-1-activator protein-1 site)

Dominant negative inhibitors identify the functional requirement of both the RD and AP-1 sites for TGF-β1-stimulated collagenase-3 promoter activity - To determine the functional role of the RD sites and its transcription factor family, Cbfa, for TGF-β1-stimulated collagenase-3 promoter activity in MDA-MB231 cells, we used AML/ETO, a chimeric protein caused by chromosomal translocation t(8;21) that lacks a transactivation domain at the carboxyterminal portion of AML1 and acts as a repressor of Cbfa proteins (5). Since mutations either at the distal or the proximal RD sites in the collagenase-3 promoter constructs inhibit the TGF-β1-response (Fig. 4B), the wild type collagenase-3 promoter construct (-148) was used in these studies. The collagenase-3 promoter construct was transiently co-transfected into MDA-MB231 cells with an AML/ETO expression plasmid (Fig. 5). The result demonstrates that the basal and the TGF-β1-response were greatly reduced by overexpression of AML/ETO, suggesting that both the RD site and Cbfa are required for collagenase-3 promoter activity in MDA-MB231 cells. Previously, we showed that there is a high level of expression of fra-1 in MDA-MB231 cells (11). Like c-Fos, Fra-1 also heterodimerizes with Jun family members. Hence, to identify the functional role of the AP-1 site and its transcription factors involved in TGF-β-stimulated collagenase-3 promoter activity, an antisense-Fra-1 expression plasmid was transiently transfected with the collagenase-3 promoter construct. As shown in Fig. 5, co-transfection of antisense-Fra-1 plasmid inhibited both the basal and TGF-β1-stimulated collagenase-3 promoter activity indicating that the inhibition of Fra-1 expression by antisense-Fra-1 may abolish AP-1 transactivation since Fra-1 forms a complex with c-Jun, JunB or JunD that is necessary for AP-1 transactivation.

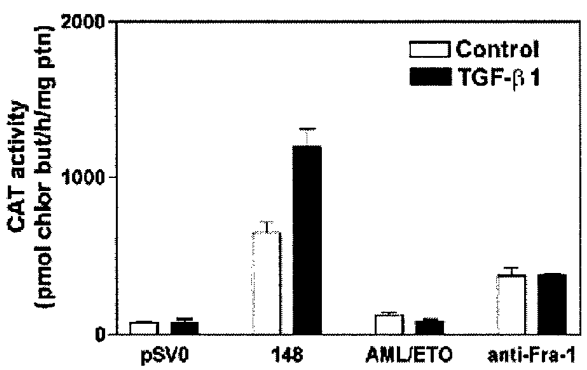


Figure. 5. Functional requirement of both the RD and AP-1 sites and their transcription factors for TGF-β1-stimulated collagenase-3 promoter activity. The wild type collagenase-3 promoter construct (-148) was transiently cotransfected with either pCMV-AML-1/ETO construct or pCMV-anti-Fra-1 construct into MDA-MB231 cells and then treated with control or TGF-β1-containing media for 24 h, and assayed for CAT activity. The total amount of DNA used for all transfection with or without the expression constructs was equalized with pCMV. Data represent mean ± S.E. of three experiments.

TGF-β1-stimulated collagenase-3 promoter activation depends on TGF-β receptors - Two different transmembrane protein serine/threonine kinases, known as receptor types I and II, are brought together by TGF-β, which acts as a receptor assembly factor. In the ligand-induced complex, receptor II phosphorylates the GS region, resulting in activation of the receptor I kinase. In order to determine the specificity of TGF-β-signaling mediated by TGF-β receptors for collagenase-3 promoter activity, the collagenase-3 promoter construct was transiently co-transfected into MDA-MB231 cells with a dominant-negative TGF-β type II receptor expression plasmid. As shown in Fig. 6A, overexpression of dominant-negative TGF-β type II receptor resulted in significant decreases in basal, as well as TGF-β1-stimulated collagenase-3 promoter activity. The reduced basal promoter activity by overexpression of dominant negative TGF-β type II receptor in MDA-MB231 cells could possibly be a result of inhibition of endogenous TGF-β signaling. To explore this possibility we used Western blot analysis to detect the expression of TGF-β1 in MDA-MB231 cells. As shown in Fig. 6B, three major bands of the sizes of 17 kDa, 45 kDa, and 65 kDa corresponding to the biologically active peptide and precursor forms of TGF-β1 were detected by a specific TGF-β1 antibody. Thus, the endogenous TGF-β1 contributes the high basal expression of collagenase-3 in MDA-MB231 cells.

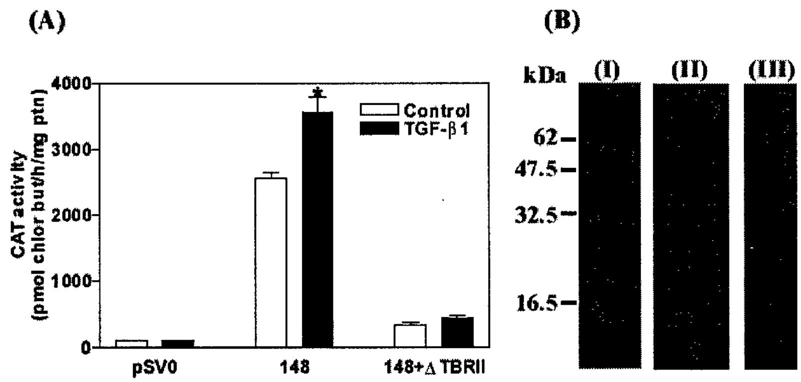


Figure 6. The TGF-β1-stimulated collagenase-3 promoter activity is mediated by TGF-β receptors. A, The wild type collagenase-3 promoter construct (-148) was transiently cotransfected with pCMV-TGF-β type II receptor mutant (TBRII) into MDA-MB231 cells for 24 h, and assayed for CAT activity. The total amount of DNA used for all transfection with or without the expression constructs was equalized with pCMV. Data represent mean ± S.E. of three experiments. *Significant difference compared with control (P< 0.001). **B,** Whole cell extracts were prepared from MDA-MB231 cells and subjected to Western blot analysis as described in methods section. Blots were incubated overnight with either IgG (I) or anti-TGF-β1 (II) or anti-TGF-β1 (III) that had been preadsorbed for 30 min with 100X peptide against which it was raised. TGF-β1 was detected by ECL system.

Key Research Accomplishments:

- The construct pCMV-MMP-1-V5 containing the human cytomegalovirus promoter sequence and a 1.65 kb MMP-1 (human matrix metalloproteinase-1 or collagenase-1) cDNA followed by a V5-epitope tag was made.
- Collagenase-3 is efficiently expressed in COS-7 cells under the control of CMV promoter.
- A transgenic construct pOC-MMP-1-V5 containing a ~1 kb rat osteocalcin promoter fragment and a 1.65 kb MMP-1 cDNA fragment followed by a V5-epitope tag was constructed.
- TGF-β1 (transforming growth factor--β1) stimulates collagenase-3 expression in the human breast cancer cell line MDA-MB231.
- TGF-β1 stimulation of collagenase-3 expression in MDA-MB231 cells requires *de novo* protein synthesis.
- The RD (runt domain binding sequence) and AP-1 (activator protein-1) sites are necessary for TGF-β1-stimulated collagenase-3 promoter activity in human breast cancer cells.
- Dominant negative inhibitors identify the functional requirement of both the RD and AP-1 sites for TGF-β1-stimulated collagenase-3 promoter activity.
- TGF-β1-stimulated collagenase-3 promoter activation depends on TGF-β receptors.

Reportable Outcomes:

Manuscript:

TGF-β1 stimulation of collagenase-3 requires the core binding factor alpha and activator protein-1 sites in human breast cancer cells
N. Selvamurugan, Z. Fung, D. T. Denhardt, and N. C. Partridge (2002)
Submitted to Journal of Biological Chemistry

Abstract:

Transforming Growth Factor-β stimulation of collagenase-3 expression in human breast cancer cells
N. Selvamurugan, Z. Fung, and N. C. Partridge
To be presented at the 24th Annual meeting of American Society for Bone and Mineral Research, San Antonio, TX on September 20-24, 2002.

Conclusions:

1. The application of a transgenic mouse model will contribute greatly to the understanding of the pathogenesis of bone metastasis. Identification of the exact nature of these tumor-bone interactions may not only generate valuable information on underlying regulatory mechanisms in invasion and bone metastasis but can also be of value in the development of therapeutic strategies.

2. A better understanding of TGF-β1-stimulated collagenase-3 expression in the metastasis of cancer cells to bone, local tumor growth and subsequent bone destruction, can lead to effective therapies by targeting this enzyme.

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Appendices: N/A